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Detection by Multiplex Real-Time Polymerase Chain Reaction Assays and Isolation of Shiga Toxin–Producing *Escherichia coli* Serogroups O26, O45, O103, O111, O121, and O145 in Ground Beef

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Abstract

Six Shiga toxin–producing *Escherichia coli* (STEC) serogroups, which include O26, O45, O103, O111, O121, and O145, are responsible for the majority of non–O157 STEC infections in the United States, representing a growing public health concern. Cattle and other ruminants are reservoirs for these pathogens; thus, food of bovine origin may be a vehicle for infection with non–O157 STEC. Methods for detection of these pathogens in animal reservoirs and in food are needed to determine their prevalence and to develop intervention strategies. This study describes a method for detection of non–O157 STEC in ground beef, consisting of enrichment in modified tryptic soy broth at 42°C, followed by real-time multiplex polymerase chain reaction (PCR) assays targeting stx_1, stx_2, and *eae* genes and the *wzx* gene in the O-antigen gene clusters of the six serogroups, and then immunomagnetic separation (IMS) followed by plating onto Rainbow® Agar O157 and PCR assays for confirmation of isolates. All ground beef samples artificially inoculated with 1–2 and 10–20 CFU/25 g of ground beef consistently gave positive results for all of the target genes, including the internal amplification control using the multiplex real-time PCR assays after enrichment in modified tryptic soy broth for a total of 24 h (6 h at 37°C and 18 h at 42°C). The detection limit of the real-time multiplex PCR assays was ~50 CFU per PCR. IMS for O26, O103, O111, and O145 was performed with commercially available magnetic beads, and the IMS beads for O45 and O121 were prepared using polyclonal antiserum against these serogroups. A large percentage of the presumptive colonies of each serogroup picked from Rainbow Agar O157 were confirmed as the respective serogroups; however, the percent recovery of STEC O111 was somewhat lower than that of the other serogroups. This work provides a method for detection and isolation in ground beef and potentially other foods of non–O157 STEC of major public health concern.

Introduction

Shiga toxin–producing *Escherichia coli* (STEC), also known as verocytotoxin-producing *E. coli*, are important foodborne pathogens responsible for outbreaks of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). STEC that cause HC and HUS are also referred to as enterohemorrhagic *E. coli* (Gyles, 2007). *E. coli* O157:H7 is the most commonly recognized STEC in the United States; however, many other STEC serogroups have been isolated from animals and food and have caused human illness. A number of these serogroups, including STEC O26, O103, O111, and O145, have been associated with outbreaks and sporadic cases of HC and HUS worldwide (Paton *et al.*, 1996; Hiruta *et al.*, 2001; Hoshina *et al.*, 2001; McCarthy *et al.*, 2001; McMaster *et al.*, 2001; Werber *et al.*, 2002; Misselwitz *et al.*, 2003; Brooks *et al.*, 2004; Ethelberg *et al.*, 2007; Gyles, 2007). Cattle are the most important reservoir for *E. coli* O157:H7 and other STEC strains, and food of bovine origin or food or water contaminated with bovine feces has been linked to HC and HUS (Smith and Fratamico, 2005; Hussein, 2007). In 1994, the Food Safety and Inspection Service, U.S. Department of Agriculture, declared *E. coli* O157:H7 an adulterant in beef products and began a verification sampling program to test for the pathogen in samples collected from federally inspected establishments and retail stores. These efforts have proven to be effective in reducing the risk of infection with *E. coli* O157:H7.

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STEC possess a number of virulence factors, and the production of Shiga toxins (Stx1 and/or Stx2) is the most critical. STEC also possess a pathogenicity island called the locus of enterocyte effacement, which encodes for proteins necessary for the formation of attaching and effacing lesions, including intimin (Eae), an outer membrane protein, a translocated intimin receptor (Tir), a type III secretion apparatus, and effector proteins translocated by the secretion system. Non–O157 STEC strains that carry both the stx_2 and the *eae* genes were more often associated with severe disease, including HUS, independent of serogroup, and the importance of stx_2 and *eae* was also demonstrated using an infant rabbit animal model (Boerlin *et al.*, 1999; Ritchie *et al.*, 2003; Werber *et al.*, 2003). Several other candidate pathogenicity islands, including OI (O-island)-57, OI-71, and OI-122 found in STEC correlated independently with outbreak and HUS potential of non–O157 STEC (Coombes *et al.*, 2008). However, the role of virulence genes carried on these pathogenicity islands in disease is not fully clear. STEC also carry genes for a hemolysin encoded in the *hly* operon found on the virulence plasmid, and for various adhesins and cytotoxins, which may also contribute to virulence (Gyles, 2007).

Twenty percent to 70% of STEC infections throughout the world are due to non–O157 STEC (WHO, 1998). In 2000, CDC's FoodNet surveillance program began collecting data on non–O157 STEC, and the Council of State and Territorial Epidemiologists recommended that non–O157 STEC that cause human illness become reportable to the Nationally Notifiable Diseases Surveillance System. Consequently, reporting of non–O157 STEC by public health departments has increased. A review of the records for non–O157 STEC isolates forwarded by state public health laboratories to the CDC's reference laboratory between 1983 and 2002 for confirmation and serotyping showed that six serogroups, O26, O45, O111, O103, O121, O45, and O145, of the 61 serogroups identified accounted for 71% of the isolates recovered (Brooks *et al.*, 2005).

Determining the true incidence of disease caused by non–O157 STEC is problematic since it is important to detect the presence of the Shiga toxins or the *stx* genes and other virulence genes, and then it is necessary to isolate the strain and determine the serotype. Unlike *E. coli* O157:H7, most non–O157 STEC cannot be easily distinguished from nonpathogenic strains using commercially available selective and differential media. A plating medium consisting of washed sheep's blood agar containing mitomycin C enhanced the ability to detect enterohemolysin-producing O157:H7 and non–O157 STEC strains (Sugiyama *et al.*, 2001); however, STEC strains that do not produce the hemolysin will not be detected. Therefore, there is a need for methods for detection, identification, and isolation of important non–O157 STEC serogroups to determine the true incidence of human infections caused by these pathogens and their prevalence in food.

The objective of this study was to develop a method for detection and isolation of the top six non–O157 STEC serogroups: O26, O45, O103, O111, O121, and O145. The method described in this report involves screening enrichments using multiplex polymerase chain reaction (PCR) assays targeting virulence genes and the *wzx* gene in the O-antigen gene clusters of the six serogroups followed by immunomagnetic separation (IMS), plating onto selective/differential agar, and PCR assays for confirmation of isolates.

Materials and Methods

Bacteria

E. coli strains used in this study and their sources are listed in Table 1. A total of 29 STEC belonging to serogroups O26, O45, O103, O111, O121, and O145 were used for artificial inoculation of ground beef samples. The bacteria were stored at −70°C in tryptic soy broth (TSB; Becton Dickinson and Company) with 10% glycerol (Sigma-Aldrich Corp.). Working cultures were made by inoculating from the frozen stocks into TSB and incubating for 19 h at 37°C.

Inoculation and enrichment of ground beef samples

Ground beef (15% fat) obtained from local markets was used on the day of purchase. Overnight cultures were diluted in 0.1% peptone (Becton Dickinson), and 25 g of the ground beef samples were inoculated with 2 mL of diluted cultures, resulting in 1–2 and 10–20 CFU/25g. The diluted cultures were plated onto tryptic soy agar (TSA; Becton Dickinson) and incubated at 37°C for 18 h, and then colonies were enumerated. The medium used for enrichment was modified TSB (mTSB) as described by Possé *et al.* (2008a) with modifications. Briefly, 25-g portions of the ground beef were placed into filter Stomacher bags (VWR), and

Table 1. Serotype and Virulence Profiles of Shiga Toxin–Producing *Escherichia coli* Strains Used for Artificial Inoculation of Ground Beef

Strain	*Serotype*	*Source*	stx_1	stx_2	eae
SJ1	O26:H11	CDC[a]	+	−	+
SJ2	O26:H11	CDC	+	+	+
SJ3	O26:H11	CDC	−	+	+
00971	O26:H11	FDA[b]	+	−	+
05-6544	O26:H11	PHAC[c]	+	−	+
SJ7	O45:H2	CDC	+	−	+
SJ8	O45:H2	CDC	+	−	+
SJ9	O45:H2	CDC	+	+	+
05-6545	O45:H2	PHAC	+	−	+
SJ10	O103:H2	CDC	+	−	+
SJ11	O103:H25	CDC	+	−	+
SJ12	O103:H11	CDC	+	+	+
04162	O103:H6	FDA	+	−	+
04-3973	O103:H11	PHAC	+	−	+
00-4748	O111:NM	PHAC	+	+	+
98-8338	O111:NM	PHAC	+	−	+
01387	O111:H8	FDA	+	−	+
SJ13	O111:NM	CDC	+	+	+
SJ14	O111:H8	CDC	+	+	+
SJ15	O111:NM	CDC	+	−	+
SJ16	O121:H19	CDC	−	+	+
08023	O121:H19	FDA	−	+	+
SJ18	O121:H19	CDC	+	+	+
03-2832	O121:H19	PHAC	−	+	+
SJ23	O145:NM	CDC	+	+	+
SJ24	O145:NM	CDC	−	+	+
SJ25	O145:H25	CDC	−	+	+
07865	O145:H18	FDA	−	+	+
03-4699	O145:NM	PHAC	+	−	+

[a]CDC, Centers for Disease Control and Prevention, Atlanta, GA.
[b]FDA, Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, MD.
[c]PHAC, Public Health Agency of Canada, Winnipeg, Manitoba, Canada.

225 mL of TSB containing 10 mg/L of cefsulodin and 16 mg/L of vancomycin (Sigma-Aldrich Corp.) were added followed by pummeling for 1 min using a Stomacher lab blender (Cooke Laboratory Products). The samples were incubated static for 6 h at 37°C. After this 6-h pre-enrichment step, bile salts (1.5 g/L; Difco Laboratories), rifampicin (2.0 mg/L), and potassium tellurite (1.0 mg/L; Sigma-Aldrich) were added, and incubation was continued for 18 h at 42°C. As a negative control, an uninoculated ground beef sample was also subjected to enrichment. Aerobic plate counts of uninoculated ground beef samples were determined by preparing dilutions in 0.1% peptone and plating onto TSA. After incubation for 18 h at 37°C, the colonies were enumerated.

DNA extraction

One milliliter of the enrichments was transferred to a microcentrifuge tube, and the cells were harvested by centrifugation at 16,000 *g* for 3 min. The supernatant was removed, and the pellet was resuspended in 200 μL of the PrepMan Ultra reagent (Applied Biosystems). The tubes were heated in a boiling water bath for 10 min, cooled to room temperature, and then centrifuged for 3 min at 16,000 *g*. The supernatant, which was used as template for the real-time multiplex PCR assays, was transferred to a sterile microcentrifuge tube or was stored at −20°C when not used immediately.

Real-time multiplex PCR assays

Omnimix™ reagent beads (Fisher Scientific) were rehydrated as recommended by the manufacturer, and per 25 μL reaction consisted of 1.5 U TaKaRa hot start *Taq* polymerase, 200 μM dNTP, 4 mM $MgCl_2$, and 25 mM HEPES, pH 8.0. One microliter of template DNA was used per reaction. The sequences of the primers and probes and the dyes used for the TaqMan probes, the concentrations of each primer and probe (Integrated DNA Technologies, Inc.) used in the multiplex PCR assays, and the expected sizes of the PCR products are shown in Table 2.

Two internal controls were evaluated in the multiplex PCR assays. The first targeted the 16S rRNA gene of gammaproteobacteria, yielding a 99-bp PCR product (Fratamico *et al.*,

Table 2. Oligonucleotide Primers and Probes Used in the Real-Time Polymerase Chain Reaction Assays

Primers and probes	*Sequence (5′–3′)*	*Concentration (μM)*	*Target gene*	*Product size (bp)*
stx1-150-F	GACTGCAAAGACGTATGTAGATTCG	0.25	stx_1	150
stx1-150-R	ATCTATCCCTCTGACATCAACTGC	0.25		
stx1-150-P	Texas Red-TGAATGTCATTCGCTCTGCA ATAGGTACTC-Iowa Black	0.1875		
stx2-200-F	ATTAACCACACCCCACCG	0.25	stx_2	200
stx2-200-R	GTCATGGAAACCGTTGTCAC	0.25		
stx2-200-P	Texas Red-CAGTTATTTTGCTGTGGATAT ACGAGGGCTTG-Iowa Black	0.1875		
Eae170-F	CTTTGACGGTAGTTCACTGGAC	0.25	*eae*	170
Eae170-R	CAATGAAGACGTTATAGCCCAAC	0.25		
Eae170-P	FAM-CTGGCATTTGGTCAGGTCGGGGCG-BHQ1	0.1875		
Wzx158-O26-F	GTATCGCTGAAATTAGAAGCGC	0.25	O26 *wzx*	158
Wzx158-O26-R	AGTTGAAACACCCGTAATGGC	0.25		
Wzx158-O26-P	FAM-TGGTTCGGTTGGATTGTCCATAAGAGGG-BHQ1	0.20		
Wzx237-O111-F	TGTTCCAGGTGGTAGGATTCG	0.25	O111 *wzx*	237
Wzx237-O111-R	TCACGATGTTGATCATCTGGG	0.25		
Wzx237-O111-P	Texas Red-TGAAGGCGAGGCAACACATTA TATAGTGC-Iowa Black	0.20		
Wzx72-O45-F	CGTTGTGCATGGTGGCAT	0.25	O45 *wzx*	72
Wzx72-O45-R	TGGCCAAACCAACTATGAACTG	0.25		
Wzx72-O45-P	FAM-ATTTTTTGCTGCAAGTGGGCTGTCCA-BHQ1	0.1875		
Wzx189-O121-F	AGGCGCTGTTTGGTCTCTTAGA	0.25	O121 *wzx*	189
Wzx189-O121-R	GAACCGAAATGATGGGTGCT	0.25		
Wzx189-O121-P	Texas Red-CGCTATCATGGCGGGACAATGA CAGTGC-Iowa Black	0.1875		
Wzx191-O103-F	TTGGAGCGTTAACTGGACCT	0.25	O103 *wzx*	191
Wzx191-O103-R	ATATTCGCTATATCTTCTTGCGGC	0.25		
Wzx191-O103-P	Texas Red-AGGCTTATCTGGCTGTTCTTA CTACGGC-Iowa Black	0.20		
Wzx135-O145-F	AAACTGGGATTGGACGTGG	0.25	O145 *wzx*	135
Wzx135-O145-R	CCCAAAACTTCTAGGCCCG	0.25		
Wzx135-O145P	FAM-GCTAATTGCAGCCCTTGCACTACGAGGC-BHQ1	0.10		
IC-F	GCAGCCACTGGTAACAGGAT	0.25	pUC19	118
IC-R	GCAGAGCGCAGATACCAAAT	0.25	pMB1 *rep*	
IC-P	Cy5-AGAGCGAGGTATGTAGGCGG-BHQ2	0.20		
16SrRNA IC-F	CCTCTTGCCATCGGATGTG	0.10	16S rRNA	99
16SrRNA IC-R	GGCTGGTCATCCTCTCAGACC	0.10		
16SrRNA IC-P	Cy5-GTGGGGTAACGGCTCACCTAGGCGAC-BHQ2	0.0313		

2009a). The concentration of the 16S rRNA primers and Cy5-labeled probe used in the PCR are shown in Table 2. The second internal control that was evaluated consisted of linearized pUC19 (New England Biolabs), and the sequences of the primers and probe targeting pMB1 *rep* gene (118-bp product) and the concentrations used are shown in Table 2. pUC19 was linearized by digestion with *Eco*RI (37°C for 2 h) and extracted from an agarose gel using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The DNA, eluted using Buffer EB, was diluted in TE buffer, pH 8.0, to a concentration of 10^4 copies per microliter. One microliter was used in the multiplex PCR assays.

The four multiplex PCR assays used were (1) primers and probes targeting the stx_1, stx_2, and *eae* genes and the internal control; (2) the *E. coli* O26 and O111 *wzx* genes and the internal control; (3) the *E. coli* O103 and O145 *wzx* genes and the internal control; and (4) the *E. coli* O121 and O45 *wzx* genes and the internal control. The multiplex PCR assays were performed using a SmartCycler (Cepheid), and the cycling conditions for all of the multiplex PCR assays consisted of an initial DNA denaturation step at 94°C for 2 min followed by 40 cycles of 94°C for 20 sec, 60°C for 30 sec, and 72°C for 50 sec.

Sensitivity of the multiplex PCR assays

The sensitivity of the PCR assays was determined for *E. coli* serogroups O26, O45, O103, O111, O121, and O145. Ten-fold serial dilutions of overnight cultures were made in 0.1% peptone, and dilutions (100 μL) were added to 900-μL aliquots of uninoculated control enrichments to obtain samples containing 10^7 to 10^1 CFU/mL. Only one strain of each serogroup was inoculated to determine sensitivity, and the concentration of the bacteria was verified by plating onto TSA. DNA extraction and the multiplex PCR assays were performed as described above.

Preparation of magnetic beads for IMS of E. coli *O45 and* E. coli *O121*

Magnetic beads for IMS of *E. coli* O45 and *E. coli* O121 are not commercially available; therefore, these were prepared using polyclonal anti-O45 and anti-O121 antisera. The typing sera were kindly provided by Dr. Chitrita DebRoy at the *E. coli* Reference Center at the Pennsylvania State University. IgG was purified from the rabbit antiserum using Protein G Plus Agarose (Cat. No. 22851; Thermo Fisher Scientific) based on the method published by Medina (2006). The IgG was then biotinylated using the EZ-Link Maleimide-PEO Solid Phase Biotinylation Kit (Cat. No. 21930; Thermo Fisher Scientific) according to the manufacturer's instructions. After determining the protein concentration of the biotinylated anti-O45 and anti-O121 antisera using the Bio-Rad Protein Assay (Bio-Rad), Dynabeads M-280 Streptavidin (Invitrogen; Cat. No. 112.05D) were coated with the biotinylated antibodies according to the manufacturer's instructions. The beads were stored at 4°C.

IMS and plating onto Rainbow® Agar O157

E. coli O26, O45, O103, O111, O121, and O145 strains were recovered from enrichments of artificially inoculated ground beef samples by IMS. Twenty microliters of Dynabeads EPEC/VTEC O26, O103, O111, O145 (Invitrogen), or Dynabeads M-280 streptavidin with biotinylated *E. coli* O45 and *E. coli* O121 antibodies were mixed with 1 mL of the enrichments in a 1.5 mL microcentrifuge tube. The tubes were placed on a rotator for 10 min, before collecting the beads using a Dynal magnetic particle concentrator, and then the supernatant was removed. The beads were washed three times using 1 mL of phosphate-buffered saline containing 0.05% Tween 20 (PBS-T; Sigma-Aldrich), and then were resuspended in 100 μL PBS-T. As recommended by the manufacturer, a sterile swab was used to streak the Dynabeads onto Rainbow Agar O157 (Biolog, Inc.), and the plates were incubated at 37°C for 24 h. Colonies for confirmation by the PCR assays were picked from Rainbow Agar O157 plates based on the typical color of the colonies of the different serogroups (Table 3).

Confirmation of presumptive isolates

One to five presumptive *E. coli* O26, O45, O103, O111, O121, or O145 colonies were picked from each Rainbow Agar O157 plate, and the colonies were resuspended in 100 μL of sterile water and lysed by heating at 99°C for 10 min. The multiplex PCR assays targeting the stx_1, stx_2, and *eae* genes and the O-serogroup-specific *wzx* genes were performed using 1 μL of the lysates as described above to confirm the identity of the colonies.

Results and Discussion

Detection and isolation of STEC other than serogroup O157 from food and other types of samples is problematic due to the lack of differential phenotypic characteristics from nonpathogenic *E. coli*. The development of molecular reagents capable of identifying both virulence genes and serogroup-specific genetic determinants holds promise for a more comprehensive characterization of food samples to determine the prevalence of specific non–O157 STEC in food and to establish the incidence of human infections caused by these emerging

Table 3. Isolation of Non–O157 Shiga Toxin–Producing *Escherichia coli* on Rainbow Agar O157

		No. of colonies confirmed as correct serogroup/no. of colonies picked (%)	
E. coli *serogroup*	*Colony color on Rainbow Agar O157*	*Immunomagnetic separation*	*Plated without immunomagnetic separation*
O26	Purple	15/21 (71%)	7/12 (58%)
O45	Gray-purple, purple, light magenta	23/27 (85%)	14/22 (68%)
O103	Gray, purple, blue-purple	18/20 (90%)	10/12 (83%)
O111	Gray-blue, gray	8/44 (18%)	8/27 (30%)
O121	Magenta, purple	22/27 (81%)	12/24 (50%)
O145	Gray-purple, blue-purple	17/20 (85%)	9/10 (90%)

pathogens. Six non–O157 STEC serogroups (O26, O45, O103, O111, O121, and O145) are responsible for the majority of non–O157 STEC infections in the United States, and outbreaks caused by these pathogens have been linked to a variety of foods, drinking and recreational water, and animal contact (Brooks *et al.*, 2005). Similar to STEC O157:H7, cattle are an important reservoir for non–O157 STEC, and beef products and food potentially contaminated with animal feces have been associated with human illness (Smith and Fratamico, 2005; CDC Outbreak Surveillance Data, www.cdc.gov/foodborneoutbreaks/outbreak_data.htm).

The stx_1, stx_2, and *eae* genes are important STEC virulence markers, and PCR assays for detection of *E. coli* O157:H7 and non–O157 STEC have targeted these and other virulence genes (Fratamico *et al.*, 2000; Paton and Paton, 2002; Fratamico and DebRoy, 2010). Further, multiplex PCR assays targeting STEC virulence genes and genes in the O-antigen gene clusters of STEC serogroups have also been described (Fratamico *et al.*, 2003; DebRoy *et al.*, 2004; Perelle *et al.*, 2004; Fratamico *et al.*, 2005; Monday *et al.*, 2007; Fratamico *et al.*, 2009b). In the current study, an approach was designed to detect the top six non–O157 STEC serogroups (O26, O45, O103, O111, O121, and O145), which involved (1) enrichment in mTSB; (2) DNA extraction followed by multiplex PCR assays targeting the stx_1, stx_2, and *eae* genes; (3) multiplex PCR assays targeting the *wzx* genes in the O-antigen gene clusters of the six serogroups; (4) IMS; (5) plating onto Rainbow Agar O157; and (6) confirmation of presumptive positive colonies using the multiplex PCR assays.

Enrichment of ground beef samples

E. coli O26, O45, O103, O111, O121, and O145 strains (Table 1) inoculated into 25-g samples of ground beef were subjected to enrichment in the mTSB medium described by Possé *et al.* (2008a). When multiplex PCR assays were performed as described below, *E. coli* O111 did not generate a fluorescence signal, or the signal was very weak. It was ascertained that novobiocin (8 mg/L) in the mTSB medium slowed the growth of STEC O111 strains. The level of STEC O111 strains was ~2–3 $\log_{10}$ CFU/mL lower when grown in mTSB that contained novobiocin as one of the selective agents compared to growth in TSB (data not shown). Therefore, novobiocin was not added, and instead cefsulodin at 10 mg/L was included in the mTSB. Vimont *et al.* (2007) also reported that non–O157 STEC strains were inhibited to a larger extent than *E. coli* O157:H7 strains in the presence of novobiocin in the enrichment medium. They indicated that addition of novobiocin at 20 mg/L could lead to false negative results when testing for non–O157 STEC in food. Cefsulodin inhibits the growth of *Aeromonas* and some *Pseudomonas* species, and it is frequently used in enrichment media for detection of *E. coli* (Weagant *et al.*, 1995; Kannan *et al.*, 2010). The presence of the other selective agents did not inhibit the growth of the non–O157 STEC serogroups as was shown by inoculation of 25 g of sterile ground beef with the STEC strains in mTSB and TSB (data not shown). The aerobic plate counts in the ground beef samples used for artificial inoculation were determined by plating an uninoculated sample before enrichment and were between 10^5 and 10^6 CFU/g.

Real-time multiplex PCR assays

The strategy for detection of the six non–O157 STEC serogroups in ground beef was to use multiplex real-time PCR assays targeting stx_1, stx_2, and *eae* to test enrichments as a rapid screen to quickly rule out negative samples. Samples that are positive for the stx_1 and/or stx_2 and the *eae* genes are then subjected to the serogroup-specific multiplex real-time PCR assays targeting the *wzx* genes in the O-antigen gene clusters of the six serogroups. Three multiplex PCR assays are performed with each targeting the *wzx* genes of two serogroups (O26 and O111; O103 and O145; and O121 and O45). Enrichments (stx_1/stx_2- and *eae*-positive) that are positive for one of the serogroups are then subjected to IMS and plating as described below. All of the assays were designed to have the same amplification conditions. All samples inoculated with 1–2 and 10–20 CFU/25 g of ground beef consistently gave positive results using the multiplex real-time PCR assays after enrichment in mTSB. Two internal amplification controls were evaluated with each assay. The first consisted of the use of primers and probe targeting the 16 rRNA gene of gamma proteobacteria (99-bp product) (Fratamico *et al.*, 2009a). The other internal system evaluated employed linearized pUC19 and primers and probe targeting the pMB1 *rep* gene carried on the plasmid (118-bp product). Both internal controls worked equally well generating a signal in the Cy5 channel of the SmartCycler. The detection limit of the PCR assays was determined by adding dilutions of the non–O157 STEC strains to aliquots of uninoculated ground beef enrichments and performing the multiplex real-time PCR assays after DNA extraction. A signal from each of the PCR products, including the internal control, was obtained consistently with samples that had been inoculated with 10^4 CFU/mL of enrichment; therefore, the detection limit per PCR was ~50 CFU. Since the cell pellet from 1 mL of enrichment was processed in 200 μL of the PrepMan Ultra reagent, this resulted in fivefold concentration, and 1 μL was used for the PCR assays; therefore, the detection limit was calculated to be ~50 CFU per PCR. These results are similar to those of Amoako *et al.* (2010), who reported a detection limit of 13–220 CFU in pure cultures of *Y. pestis* and 10^2–10^5 CFU/g in ground beef (without preenrichment). Perelle *et al.* (2007) used a PCR-ELISA targeting the stx_1 and stx_2 genes to screen food samples enriched in modified EC medium with novobiocin. This was followed by a multiplex real-time PCR assay targeting O-antigen-specific genes of *E. coli* serogroups O26, O103, O111, O145, and O157. Finally, singleplex PCR assays were performed to identify the *E. coli* serogroup. A method for isolation of the STEC strain and the confirmation protocol were not described. Twenty-one percent and 15% of raw milk ($n = 205$) and meat samples ($n = 300$), respectively, were positive by the PCR-ELISA; however, the authors commented that isolation of the STEC strain from the food samples is important to confirm the presence of the pathogen.

IMS and isolation of non–O157 STEC

Enrichments were subjected to IMS, and then the beads were plated onto Rainbow Agar O157. By and large, it was not difficult to identify and confirm the target STEC serogroups, based on the color of the colony (Table 3). When testing naturally contaminated samples, plating would be performed on enrichments in which the serogroup of the contaminating STEC would have been identified by the O-group-specific PCR assay; therefore, isolation of the STEC strain should not be problematic. Other agars, including ChromAgar O157

(Durso and Keen, 2007) and washed sheep blood agar (Sugiyama *et al.*, 2001), have also been evaluated for isolation of STEC, and further studies will also investigate the suitability of these selective/differential agars. For comparison, enrichments from some experiments were plated directly onto Rainbow Agar without IMS. Generally, there was much higher background when plating enrichments without performing IMS, and it was more difficult to identify the presumptive non–O157 STEC colonies. However, it was possible to select the target STECs from plates that were not subjected to IMS. For example, 23 out of 27 (85%) colonies picked from Rainbow Agar plates after IMS from samples inoculated with O45 were confirmed, whereas 14 out of 22 (68%) colonies were positive from samples plated without IMS (Table 3). One reason to explain why it was possible to isolate the non–O157 STECs without IMS is that incubation in mTSB at 42°C is sufficiently selective to suppress the growth of some of the background flora. Confirmation of presumptive positive colonies was performed by using the same real-time multiplex PCR assays that were used for screening enrichments.

Isolation of *E. coli* O111 from mTSB enrichments after IMS was somewhat more problematic than the other serogroups. Out of 44 presumptive colonies from samples that were subjected to IMS, 8 (18%) were confirmed by the PCR assays. It is possible that the antibody used with the commercial Dynabeads EPEC/VTEC O111 has low affinity for *E. coli* serogroup O111. Our results agree with those of Verstraete *et al.* (2010), who reported that the percent recovery of STEC O111 in contaminated fecal samples after IMS performed with Dynabeads EPEC/VTEC O111 was low, whereas there was a greater ability to isolate STEC O26, O103, and O157. These investigators also suggested that the affinity of the O111 antibodies coating the beads may be low; however, they used the medium described by Possé *et al.* (2008b) containing novobiocin for enrichment, which may also have prevented optimal growth of STEC O111. Further, they also found that under certain conditions, significant differences in the isolation rate from fecal enrichments that were plated directly compared to those subjected to IMS before plating were not observed with non–O157 STEC. Current investigations are examining the ability of our non-O157 detection protocol to detect and isolate the non–O157 STEC serogroups from naturally contaminated ground beef and other types of food.

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Disclosure Statement

No competing financial interests exist.

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